Continual feeding of two types of microalgal biomass affected protein digestion and metabolism in laying hens

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ABSTRACT: A 14-wk study was conducted to determine the nutritional efficacy and metabolic impact of 2 types of microalgal biomass as alternative protein sources in laying hen diets. Shaver hens (total = 150 and 26 wk old) were fed 1 of 5 diets: a control or a defatted green microalgal biomass (DG; Desmodesmus spp.) at 25% and a full-fatted diatom biomass (FD; Staurosira spp.) at 11.7% inclusion with or without protease. This experiment consisted of 5 replicates per treatment and each replicate contained 6 hens individually reared in cages (1 hen for biochemical data/replicate). Despite decreased ADFI (P = 0.03), hens fed DG or FD had final BW, overall hen–day egg production, and egg quality similar to the controls. Feeding DG or FD did not alter plasma concentrations of insulin, glutamine, and uric acid or alkaline phosphatase activity at wk 8 or 14 but decreased plasma 3-methylhistidine concentrations (P = 0.03) and tartrate-resistant acid phosphatase (TRAP) activities (P < 0.001) at wk 14 and improved ileal total AA digestibility. Although DG or FD exhibited moderate effects on intestinal brush border protease activities and mRNA levels of duodenal transporters Pept1, Lat1, and Cat1, both substantially enhanced phosphorylation of hepatic protein synthesis key regulator S6 ribosomal protein (S6) and the ratio of phospho-S6 to S6 in the liver of hens. However, DG and FD manifested with different impacts on weights of egg and egg albumen, proteolytic activity of jejunal digesta, plasma TRAP activity, ileal total AA digestibility, and several intestinal genes and hepatic proteins. Supplemental protease in the DG and FD diets produced mixed effects on a number of measures. In conclusion, our findings revealed the feasibility of including greater levels of microalgal biomass as a source of feed protein for laying hens and a novel potential of the biomass in improving dietary protein digestion and body protein metabolism than previously perceived.

Key words: egg, hens, metabolism, microalgae, protein

INTRODUCTION

Feed applications of algae were reported earlier (Becker, 2004), and approximately 5,000 t of biomass is produced each year (Spolaore et al., 2006). Recently heightened interest in microalgae for biofuel production (Gouveia et al., 2008) may allow defatted microalgal biomass to become a new feed ingredient. In an earlier 8-wk study (Leng et al., 2014), our laboratory found that laying hens tolerated 7.5 but not 15% of defatted diatom (Staurosira spp.) microalgae. Compared with those fed the control diet, hens fed 15% of the microalgal biomass had lower concentration of plasma uric acid, the primary N excretion product in birds (Vit et al., 1993). The inclusions of 7.5 or 15% microalgal biomass also altered egg albumen measures. It seems that supplemental dietary microalgae were used for body protein metabolism in the hens. Functional expressions of intestinal AA/peptide transporters and hepatic signaling of protein synthesis are closely related to dietary protein quality and body protein metabolism (Gilbert et al., 2007; Speier et al., 2012). Specifically, the main transporter systems include the cationic amino acid transporter-1 (CAT1) family, the oligopeptide transporter 1...
(PEPT1), and the Na^{+}-independent branched chain and amino acid transporter 1 (LAT1), along with functionally related intestinal aminopeptidase N (APN; Gilbert et al., 2008, 2010). Phosphorylation of S6 ribosomal protein (S6) and its upstream regulators, including P70 S6 kinase 1 (P70) and the mammalian target of rapamycin (mTOR), along with the eukaryotic initiation factor 4E (eIF4E), play important roles in the initiating protein synthesis and are responsive to dietary protein changes (Speier et al., 2012).

However, systematic impacts of different types of microalgal biomass on the nutritional and molecular aspects of protein metabolism and long-term animal production performance and health status were not studied in our previous (Leng et al., 2014) and other similar experiments (Blum and Calet, 1975; Lipstein et al., 1980; Ginberg et al., 2000; El-Deek and Al-Harthi, 2009; Halle et al., 2009; El-Deek et al., 2011; El-Deek et al., 2011). Therefore, we used defatted green microalgal biomass (DG; *Desmodesmus* spp.) and full-fatted diatom biomass (FD; *Staurosira* spp.) and conducted a layer hen study for 14 wk. The objective was to determine how continual feeding of these 2 microalgae affected long-term egg production and quality, physiological status, and protein digestion, metabolism, and the related regulatory mechanisms. Because exogenous hydrolytic enzymes may aid in the digestion of microalgae (Al-Harthi and El-Deek, 2011), we also compared effects of adding a commercially available protease into the 2 microalgal diets.

**MATERIALS AND METHODS**

Our animal protocol was approved by the Institutional Animal Care and Use Committee at Cornell University (Ithaca, NY).

**Animals, Diets, and Management**

A total of 150 (26 wk old) Shaver White commercial laying hens were randomly assigned to 5 dietary treatments. This experiment consisted of 5 replicates per treatment and each replicate contained 6 hens individually reared in cages unless otherwise indicated. The cages (29 by 47 by 44.5 cm) were equipped with nipple drinkers and individual trough feeders. Hens were provided a light:dark cycle of 16:8 h and given free access to feed and water.

The 2 types of microalgal biomass, DG and FD, were generated from a biofuel production research facility (Cellana, Kailua-Kona, HI). Proximate analyses of DG and FD (Dairy One Cooperative, Inc., Ithaca, NY) and their AA profiles (Agricultural Experiment Station Chemical Laboratories, University of Missouri, Columbia, MO) are presented in Table 1. The 5 dietary treatments were a control and diets including 25% DG or 11.7% FD supplemented without or with a protease (Ronozyme ProAct L; DSM Nutritional Products Inc., Parsippany, NJ; Table 2). All diets were formulated to be isocaloric and isonitrogenous based on analyzed values of main ingredients to meet the NRC (1994) recommendations for the laying hen. To avoid a possible masking of potential negative effects of the microalgal inclusion by high baseline levels of dietary CP and AA,

<table>
<thead>
<tr>
<th>Item</th>
<th>DG</th>
<th>FD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximate composition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moisture</td>
<td>4.0</td>
<td>14.2</td>
</tr>
<tr>
<td>Crude fat</td>
<td>1.5</td>
<td>9.3</td>
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<tr>
<td>CP</td>
<td>31.2</td>
<td>13.9</td>
</tr>
<tr>
<td>Ash</td>
<td>17.1</td>
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<tr>
<td>ADF</td>
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</tr>
<tr>
<td>NDF</td>
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<td>16.0</td>
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<tr>
<td>Mineral</td>
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<td></td>
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</tr>
<tr>
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</tr>
<tr>
<td>Mg, %</td>
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</tr>
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<td>K, %</td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>Zn, mg/kg</td>
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<td>16</td>
</tr>
<tr>
<td>Cu, mg/kg</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>Mn, mg/kg</td>
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<td>Mo, mg/kg</td>
<td>2.4</td>
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</tr>
<tr>
<td>Se, mg/kg</td>
<td>0.12</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>AA, %</td>
<td></td>
<td></td>
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<tr>
<td>Ala</td>
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</tr>
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<tr>
<td>Asp</td>
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<tr>
<td>Cys</td>
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<td>0.19</td>
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<tr>
<td>Glu</td>
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<td>1.29</td>
</tr>
<tr>
<td>Gly</td>
<td>1.72</td>
<td>0.67</td>
</tr>
<tr>
<td>His</td>
<td>0.50</td>
<td>0.18</td>
</tr>
<tr>
<td>Ile</td>
<td>1.10</td>
<td>0.55</td>
</tr>
<tr>
<td>Leu</td>
<td>2.29</td>
<td>0.94</td>
</tr>
<tr>
<td>Lys</td>
<td>1.61</td>
<td>0.57</td>
</tr>
<tr>
<td>Met</td>
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<td>0.26</td>
</tr>
<tr>
<td>Pro</td>
<td>2.73</td>
<td>0.45</td>
</tr>
<tr>
<td>Ser</td>
<td>1.10</td>
<td>0.53</td>
</tr>
<tr>
<td>Thr</td>
<td>1.26</td>
<td>0.63</td>
</tr>
<tr>
<td>Trp</td>
<td>0.43</td>
<td>0.12</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.01</td>
<td>0.40</td>
</tr>
<tr>
<td>Val</td>
<td>1.59</td>
<td>0.70</td>
</tr>
</tbody>
</table>

1DG = defatted green microalgal biomass (*Desmodesmus* spp.; Cellana, Kailua-Kona, HI); FD = full-fatted diatom biomass (*Staurosira* spp.; Cellana).

2Proximate analysis was carried out by Dairy One Cooperative, Inc. (Ithaca, NY), and AA were determined by the Agricultural Experiment Station Chemical Laboratories at the University of Missouri (Columbia, MO).
Microalgal biomass in diets for hens

was calculated on a hen–day basis; the rate of lay is weekly. Eggs were collected daily and egg production was determined as the amount of enzyme that releases 1 μmol of phenylalanine from 1 μM of substrate (Suc-Ala-Ala-Pro-Phe-p-nitroaniline)/min at pH 9.0 and 37°C. The protease activity was confirmed before mixing diets.

Body weights of the laying hens were recorded biweekly by replicates. Eggs collected on the last 3 d of the 2nd, 4th, 6th, 8th, 10th, 12th, and 14th wk were individually weighed. The same eggs were then subsequently broken and the yolks and albumens were separated and weighed and the egg shell was rinsed in distilled water, air dried, and weighed. At the end of the 8th and 14th wk, 1 hen per replicate (n = 5/treatment) was randomly selected for blood sampling via the right wing vein and euthanized via CO₂ asphyxiation to collect liver, tissue (brush border membrane) of the distal 5 cm of duodenum, jejunum, and ileum, and digesta in the duodenum, jejunum, and ileum.

**Total Amino Acid Digestibility**

Five days before euthanizing at wk 14, 1 hen per replicate (the same hens as described before for the 14th wk sample collection; n = 5/treatment) was randomly selected and fed her designated diet with the inclusion of 0.3% chromium oxide (an indigestible marker) for determination of ileal and total or excreta AA digestibility (Ravindran et al., 1999; Kim and Corzo, 2012). Excreta was collected for 5 d. At the end of collection, the hens were euthanized via CO₂ asphyxiation to collect digesta and tissue samples (as described before). Total AA concentrations in excreta, ileal digesta, and feed were determined (Phthalaldehye Reagent Complete Solution; Sigma-Aldrich Co. LLC, St. Louis, MO). All chemicals used in this study were purchased from the same company (Sigma-Aldrich Co. LLC) unless otherwise indicated.

**Plasma Biochemistry**

Blood samples were centrifuged at 1,000 × g for 15 min at 4°C. The plasma fraction was separated and frozen at −20°C. High performance liquid chromatography was used to assay for plasma concentrations of insulin (Sarmento et al., 2006), 3-methylhistidine (3-MH; Henrikson and Meredith, 1984), corticosterone (Fowler et al., 1983), and glutamine (Georgi et al., 1993). Plasma uric acid concentrations were determined using the uric acid liquid stable reagent kit (Infinity TM; Fisher Diagnostics, Middletown, NY). Plasma activities of alkaline phosphatase (AKP) were assayed according to the method of Bowers and Featherston, 1976; Vit et al., 1993) were determined using the uric acid liquid stable reagent kit (Infinity TM; Fisher Diagnostics, Middletown, NY). Plasma activities of alkaline phosphatase (AKP) were assayed according to the method of Bowers and Featherston, 1976; Vit et al., 1993) were determined using the uric acid liquid stable reagent kit (Infinity TM; Fisher Diagnostics, Middletown, NY).

**Table 2. Composition and nutrient values of experimental diets**

<table>
<thead>
<tr>
<th>Ingredient, g/kg</th>
<th>Control</th>
<th>DG</th>
<th>FD</th>
<th>Protease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn, grain</td>
<td>634</td>
<td>487</td>
<td>487</td>
<td>516</td>
</tr>
<tr>
<td>Microalgal biomass</td>
<td>–</td>
<td>250</td>
<td>250</td>
<td>117</td>
</tr>
<tr>
<td>Soybean meal (48% CP)</td>
<td>200</td>
<td>50</td>
<td>50</td>
<td>184</td>
</tr>
<tr>
<td>Wheat</td>
<td>30.0</td>
<td>59.0</td>
<td>59.0</td>
<td>31.2</td>
</tr>
<tr>
<td>Corn oil</td>
<td>21.0</td>
<td>47.5</td>
<td>47.5</td>
<td>54.5</td>
</tr>
<tr>
<td>Limestone</td>
<td>88.0</td>
<td>91.5</td>
<td>91.5</td>
<td>78.0</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>15.6</td>
<td>7.1</td>
<td>7.1</td>
<td>11.7</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>3.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FeSO₄₂</td>
<td>2.2</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Choline (60%)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Lys HCl (98.5%)</td>
<td>–</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Met (99%)</td>
<td>1.1</td>
<td>1.7</td>
<td>1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>Vitamin/mineral premix²</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
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</tr>
<tr>
<td>Protease³</td>
<td>–</td>
<td>0.6</td>
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</table>

Calculated concentration, as-fed

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>DG</th>
<th>FD</th>
<th>Protease</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME, kcal/kg</td>
<td>2,800</td>
<td>2,800</td>
<td>2,800</td>
<td>2,800</td>
</tr>
<tr>
<td>CP, g/kg</td>
<td>146</td>
<td>146</td>
<td>146</td>
<td>146</td>
</tr>
<tr>
<td>Lys, g/kg</td>
<td>7.9</td>
<td>7.9</td>
<td>7.9</td>
<td>7.9</td>
</tr>
<tr>
<td>Met, g/kg</td>
<td>3.6</td>
<td>4.2</td>
<td>4.2</td>
<td>3.9</td>
</tr>
<tr>
<td>Ca, g/kg</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>Total P, g/kg</td>
<td>5.9</td>
<td>5.0</td>
<td>5.0</td>
<td>5.5</td>
</tr>
<tr>
<td>Nonphytate, g/kg P</td>
<td>3.4</td>
<td>3.4</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td>Cl, g/kg</td>
<td>3.8</td>
<td>3.8</td>
<td>3.8</td>
<td>3.8</td>
</tr>
<tr>
<td>Fe, g/kg</td>
<td>0.06</td>
<td>0.09</td>
<td>0.09</td>
<td>0.06</td>
</tr>
</tbody>
</table>

¹DG = defatted green microalgal biomass (Desmodesmus spp.; Cellana, Kailua-Kona, HI); FD = full-fatted diatom biomass (Staurosira spp.; Cellana).
²Premix provided vitamins and minerals at the following levels (per kilogram of diet): 6,500 IU vitamin A, 3,500 IU vitamin D, 25 IU vitamin E, 5 mg menadione bisulfite, 25 mg riboflavin, 25 mg d-calcium pantothenic acid, 150 mg niacin, 11 mg vitamin B₁₂ (0.1% in mannitol), 1 mg biotin, 2.5 mg folic acid, 7 mg thiamin HCl, 25 mg pyridoxine HCl, 31.4 mg CuSO₄·5H₂O, 46 μg KI, 61.5 mg MnSO₄·4H₂O, 0.13 mg Na₂SeO₃, 43.5 mg MnO, and 1.5 mg Na₂MoO₄·4H₂O.
³Ronozyme ProAct L (DSM Nutritional Products Inc., Parsippany, NJ); 75,000 units/g and included at 600 mg/kg of feed.

the experimental diets were purposely formulated to contain 14% CP to ensure that hens required all available dietary protein. The protease inclusion rate (15,000 units/kg for diets DG + FD + was based on our previous study (Austic et al., 2013). One unit of protease activity is defined as the amount of enzyme that releases 1 μmol of p-nitroaniline from 1 μM of substrate (Suc-Ala-Ala-Pro-Phe-p-nitroaniline)/min at pH 9.0 and 37°C. The enzyme activity was confirmed before mixing diets.

Body weights of the laying hens were recorded biweekly. Eggs were collected daily and egg production was calculated on a hen–day basis; the rate of lay is reported as a percent, where the number of eggs produced in a treatment group was divided by the number of hens in the group. Feed intake was recorded biweekly by replicates. Eggs collected on the last 3 d of the 2nd, 4th, 6th, 8th, 10th, 12th, and 14th wk were individually weighed. The same eggs were then subsequently broken and the yolks and albumens were separated and weighed and the egg shell was rinsed in distilled water, air dried, and weighed. At the end of the 8th and 14th wk, 1 hen per replicate (n = 5/treatment) was randomly selected for blood sampling via the right wing vein and euthanized via CO₂ asphyxiation to collect liver, tissue (brush border membrane) of the distal 5 cm of duodenum, jejunum, and ileum, and digesta in the duodenum, jejunum, and ileum.
Protease Activity of Intestinal Digesta and Brush Border and Amino Acid Digestibility

Once the selected hen was killed, her contents of duodenum, jejunum, and ileum were flushed with ice-cold PBS, placed in sterile vials, snap frozen in liquid N, and stored at −80°C. Intestinal digesta were thawed on ice, homogenized for 60 s, and centrifuged at 18,000 × g for 20 min at 4°C. The supernatant was decanted and used for the protease activity assay. Intestinal mucosal samples were collected from the 3 segments of small intestines and stored at −80°C. The mucosal samples were homogenized for 60 s and centrifuged for 15 min at 3,000 × g at 4°C. The resulting supernatant was centrifuged at 27,000 × g for 30 min at 4°C and the remaining pellet was resuspended for the protease activity determination. Total protease activity was determined using the azocasein assay method (Tomarelli et al., 1949).

Quantitative PCR and Western Blot

Tissue samples of the duodenum were homogenized and total RNA was isolated and purified using TRIzol Reagent (Life Technologies, Carlsbad, CA). The RNA concentration and quality were determined (Agilent Bioanalyzer 2100; Agilent Technologies, Santa Clara, CA). The cDNA library was generated following the manufacturer’s instructions (Superscript and Random Primer/Oligo T mixture; Life Technologies). The relative mRNA expression of the selected genes, Pept1 (NM_204365.1), Lat-1 (NM_001030579.1), Cat-1 (NM_001145490.1), and Apn (NM_204861.1), were determined by RT-qPCR (Life Technologies). The relative mRNA expression for each sample was adjusted with the control hens.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence</th>
<th>Melting temperature, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apn</td>
<td>Forward</td>
<td>5′-ACCTCATCTGTAGTCCACGAAACA</td>
<td>65.9</td>
</tr>
<tr>
<td></td>
<td>Forward</td>
<td>5′-ACCTCTATCGTCACGAGCAAA</td>
<td>65.9</td>
</tr>
<tr>
<td>Cat1</td>
<td>Forward</td>
<td>5′-CCGGCTCTCAACGGGCTCTCA</td>
<td>69.9</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-TGGCAGCAAGCAGGCAAGTA</td>
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</tr>
<tr>
<td>Lat1</td>
<td>Forward</td>
<td>5′-TGCACTGTTAGACTTGGCTGCTGTT</td>
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</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-CGCAACGTTAGACTTGGCTGCTGTT</td>
<td>60.3</td>
</tr>
<tr>
<td>Pept1</td>
<td>Forward</td>
<td>5′-AGCTATGCAGATTCACGAGACCA</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5′-ACATGCCAACAGTATCTCTCATCA</td>
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<td>βAct</td>
<td>Forward</td>
<td>5′-AGACATCAGGGGTGATGGTTGGTTT</td>
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</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-TCCAGGTTGGTACAATCCGGT</td>
<td>65.3</td>
</tr>
</tbody>
</table>

1Antibodies used for the western blot analysis (Cell Signaling Technology, Beverly, MA) were eukaryotic initiation factor 4E, mammalian target of rapamycin, p70 S6 kinase, S6 ribosomal protein, and phospho-S6 ribosomal protein (PS6). The species, isotype, and dilution factor for antibodies were human, rabbit, and 1000, respectively, for all antibodies, except PS6, in which the dilution factor was 2000.

2Apn = aminopeptidase N; Cat1 = cationic amino acid transporter-1; Lat1 = L-type amino acid transporter-1; Pept1 = peptide transporter-1; βAct = β-actin.

Liver homogenates (25 μg protein) were dissolved in SDS reducing sample buffer and boiled for 5 min before loading onto 12% (7.5% for the detection mTOR, which has a large molecular weight) SDS-PAGE reducing mini-gels (Bio-Rad Laboratories Inc., Hercules, CA). Gels were run at a constant 30 mA. Proteins in the gels were transferred onto nitrocellulose membranes using a mini-trans blot cell (Bio-Rad Laboratories Inc.) at 100 V for 60 min (75 min for mTOR). Membranes were blocked in 5% milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature on a rocking platform. After three 5-min washes with TBST, membranes were incubated with the appropriate primary antibodies (Cell Signaling Technology Inc., Danvers, MA) overnight at 4°C with constant gentle agitation. Antibodies were diluted 1:1,000 in 3% BSA TBST for p70 S6 kinase, S6, phospho-S6 ribosomal protein (PS6; 1:2,000), and eIF4E and in 3% milk TBST for mTOR. Membranes were washed 3 times in TBST before incubating for 1 h at room temperature with the goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad Laboratories Inc.) diluted 1:3,000 in 3% milk TBST. After 3 washes in TBST and 5 rinses in distilled water, membranes were incubated for 5 min at room temperature (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL) before exposing to a file (Kodak BioMax XAR Film; Carestream, Rochester, NY). The relative quantification of the target protein
Microalgal biomass in diets for hens was determined as previously described (Yan et al., 2012).

Statistical Analysis

All data except for intestinal genes and hepatic-proteins were analyzed as a randomized complete block design with the GLM procedure of SAS (8.1; SAS Inst. Inc., Cary, NC). A total of 4 orthogonal contrasts were performed to determine effects of the 2 types of microalgal biomass and protease as follows: I = control vs. DG + FD (microalgal effect), II = DG vs. FD (comparison of the 2 types of microalgal biomass), III = DG vs. DG + protease (protease effect within the DG diet), and IV = FD vs. FD + protease (protease effect within the FD diet). Because of the semiquantitative nature and the limited sample size ($n = 3$) of the mRNA and Western blot analyses, only selected treatment effects were compared directly with the respective controls (DG or FD vs. control, DG vs. DG + protease, and FD vs. FD + protease) using the student $t$ test. Data are expressed as mean ± SEM or SE, and significance was defined as $P < 0.05$ unless indicated otherwise.

RESULTS

Egg Production and Quality

Compared with hens fed the control diet, those fed DG or FD had lower ($P = 0.03$) ADFI but similar BW, hen–day egg production, and egg quality traits (Table 4). Hens fed FD had greater ($P < 0.001$) egg weight and egg albumen weight than those fed DG. Supplementation of DG with protease resulted in greater ($P = 0.04$) yolk weight.

Plasma Biochemical Indicators

Dietary microalgal biomass or protease supplementation exerted no effect on plasma concentrations of insulin, glutamine, and uric acid or plasma AKP activity at either wk 8 or 14 (Table 5). Compared with those fed the control diet, hens fed the DG or FD diet had lower plasma 3-MH concentrations ($P = 0.03$) and TRAP activities ($P < 0.001$) at wk 14. Plasma corticosterone concentration at wk 14 was decreased ($P = 0.04$) by the supplemental protease in the FD diet. Plasma TRAP activities at wk 8 were lower ($P < 0.001$) in hens fed DG than those fed FD and were affected ($P < 0.04$) by the supplemental protease in both diets.

Protease Activities of Digesta and Brush Border and Amino Acid Digestibility

Protease activities in the digesta of duodenum or ileum at either wk 8 or 14 were not affected by the dietary treatments (Table 6). However, the activity at wk 14 in jejunum of the hens fed DG was 54% greater ($P < 0.001$) than that of the hens fed FD, and that activity was decreased ($P = 0.04$) by the supplemental protease in the DG diet. Compared with the hens fed the control diet, those fed DG or FD had lower ($P = 0.04$) and greater ($P = 0.04$) protease activities at wk 14 in the duodenal and jejunal brush border membranes, respectively. The addition of protease into the DG and

Table 4. Effects of dietary microalgal biomass and protease supplementations on egg production and quality

<table>
<thead>
<tr>
<th>Item</th>
<th>Diet</th>
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<th>P-value</th>
</tr>
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<tbody>
<tr>
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<td>Control</td>
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<td>FD</td>
</tr>
<tr>
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<td>Final BW, g</td>
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<tr>
<td>ADFI, g</td>
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<td>81.2</td>
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<tr>
<td>Production, %</td>
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<td>88.9</td>
<td>84.7</td>
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<td>Egg wt, g</td>
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<td>52</td>
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<td>Yolk wt, g</td>
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<td>Albumen wt, g</td>
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<td>Shell wt, g</td>
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</table>

1Values are expressed as means ($n = 5$) of biweekly data over the whole period.
2DG = defatted green microalgal biomass (Desmodesmus spp.; Cellana, Kailua-Kona, HI); FD = full-fatted diatom biomass (Staurosira spp.; Cellana).
3Preplanned contrasts were I = control vs. DG and FD, II = DG vs. FD, III = DG vs. DG + protease, and IV = FD vs. FD + protease.
5Hen–day egg production: the rate of lay is reported as a percent, where the number of eggs produced in a treatment group was divided by the number of hens in the group.
Table 5. Effects of dietary microalgal biomass and protease supplementations on plasma biochemistry of laying hens at wk 8 and 14

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>DG</th>
<th>FD</th>
<th>SEM</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
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<tbody>
<tr>
<td>3-MH, mol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>wk 8</td>
<td>23.3</td>
<td>21.6</td>
<td>22.9</td>
<td>20.5</td>
<td>20.8</td>
<td>3.1</td>
<td>0.51</td>
<td>0.78</td>
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<tr>
<td>wk 14</td>
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<td>23.8</td>
<td>25.0</td>
<td>23.0</td>
<td>21.9</td>
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</tr>
<tr>
<td>Glutamine, mmol/L</td>
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<td></td>
<td></td>
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<td>5.9</td>
<td>8.3</td>
<td>4.5</td>
<td>5.0</td>
<td>2.9</td>
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<td>0.72</td>
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<td>3.3</td>
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<td>3.6</td>
<td>4.8</td>
<td>3.2</td>
<td>1.2</td>
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<td>Uric acid, mg/L</td>
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<td>137</td>
<td>98</td>
<td>112</td>
<td>131</td>
<td>171</td>
<td>24</td>
<td>0.45</td>
<td>0.35</td>
</tr>
<tr>
<td>wk 14</td>
<td>120</td>
<td>88</td>
<td>84</td>
<td>86</td>
<td>70</td>
<td>19</td>
<td>0.19</td>
<td>0.93</td>
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<tr>
<td>Corticosterone, μg/L</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>wk 8</td>
<td>0.14</td>
<td>0.26</td>
<td>0.21</td>
<td>0.10</td>
<td>0.29</td>
<td>0.09</td>
<td>0.75</td>
<td>0.22</td>
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<tr>
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<td>0.22</td>
<td>0.26</td>
<td>0.39</td>
<td>0.17</td>
<td>0.07</td>
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<tr>
<td>Insulin, mmol/L</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>wk 8</td>
<td>0.65</td>
<td>0.53</td>
<td>0.83</td>
<td>0.51</td>
<td>0.70</td>
<td>0.18</td>
<td>0.97</td>
<td>0.69</td>
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<tr>
<td>wk 14</td>
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<td>1.30</td>
<td>0.19</td>
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<td>0.74</td>
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<tr>
<td>TRAP, units/L</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
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<td>22.8</td>
<td>17.3</td>
<td>29.1</td>
<td>33.6</td>
<td>24.2</td>
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<td>0.01</td>
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<tr>
<td>wk 14</td>
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<td>18.2</td>
<td>25.1</td>
<td>22.8</td>
<td>24.9</td>
<td>2.9</td>
<td>0.01</td>
<td>0.32</td>
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<tr>
<td>AKP, units/L</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>wk 8</td>
<td>13.8</td>
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<td>11.2</td>
<td>12.2</td>
<td>14.7</td>
<td>1.5</td>
<td>0.58</td>
<td>0.62</td>
</tr>
<tr>
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<td>13.2</td>
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<td>12.5</td>
<td>12.0</td>
<td>0.9</td>
<td>0.68</td>
<td>0.54</td>
</tr>
</tbody>
</table>

1Values are expressed as means (n = 5/treatment) of biweekly data over the whole period.
23-MH = 3-methylhistidine; TRAP = tartrate-resistant acid phosphatase; AKP, alkaline phosphatase.
3DG = defatted green microalgal biomass (Desmodesmus spp.; Cellana, Kailua-Kona, HI); FD = full-fatted diatom biomass (Staurosira spp.; Cellana).
4Preplanned contrasts were I = control vs. DG and FD, II = DG vs. FD, III = DG vs. DG + protease, and IV = FD vs. FD + protease.

FD diets elevated (P = 0.04) duodenal brush border protease activity at wk 14. Ileal AA digestibility at wk 14 was greater (P = 0.002) in the hens fed the DG or FD than those fed the control diet. The digestibility was also greater (P = 0.04) in the hens fed DG (90.6 vs. 88.0%) than that of hens fed FD. However, the total tract (excreta) AA digestibility was unaffected by dietary treatments.

**Expression of Genes and Proteins Related to Protein Metabolism**

Compared with the hens fed the control diet, those fed DG had lower (P < 0.05) duodenal Lat1 mRNA levels, whereas those fed FD had greater (P < 0.05) Cat1 and lower (P < 0.05) Pept1 mRNA levels than those fed the control diet (Table 7). Supplemental protease in the DG diet elevated (P < 0.05) Apm and Lat1 mRNA levels, whereas supplemental protease in the FD diet decreased (P < 0.05) Cat1 and increased (P < 0.05) Pept1 mRNA levels.

The amounts of hepatic PS6 and the ratios of PS6:S6 were elevated by 4- to 9-fold (P < 0.05 or P < 0.10) in the hens fed DG or FD compared with those fed the control diet (Fig. 1), whereas the hepatic S6 protein levels were not affected by the dietary treatments. The hepatic ratio of PS6:S6 was also enhanced by the supplemental protease in the FD diet (P < 0.10). Hepatic mTOR protein level was lower (P < 0.05) in the hens fed the FD diet than those fed the control diet, and the level was further decreased (P < 0.05) by the addition of protease.

**DISCUSSION**

The present study showed that feeding hens with 25% of DG or 11.7% of FD for 14 wk did not adversely affect overall egg production, BW, or egg quality, despite decreased feed intakes. Meanwhile, feeding hens with DG or FD did not alter plasma concentrations of insulin, glutamine, and uric acid or plasma AKP activities compared with those fed the control diet. Plasma TRAP activities in hens fed the DG or FD diets were...
Table 6. Effects of dietary microalgal biomass and protease supplementations on protease activity and apparent AA digestibility

<table>
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<tr>
<th>Item</th>
<th>Diet</th>
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<th>DG</th>
<th>FD</th>
<th>Protease</th>
<th>SEM</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
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<tr>
<td>Digesta, milliunits/mg digesta (as-is basis)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum wk 8</td>
<td>317.0</td>
<td>458.4</td>
<td>374.9</td>
<td>338.6</td>
<td>491.1</td>
<td>75.0</td>
<td>0.65</td>
<td>0.44</td>
<td>0.92</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>207.2</td>
<td>217.1</td>
<td>283.5</td>
<td>257.6</td>
<td>384.7</td>
<td>64.5</td>
<td>0.71</td>
<td>0.66</td>
<td>0.48</td>
<td>0.18</td>
</tr>
<tr>
<td>Jejunum wk 8</td>
<td>314.7</td>
<td>285</td>
<td>253.1</td>
<td>348.8</td>
<td>321.7</td>
<td>60.4</td>
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<td>0.69</td>
<td>0.84</td>
<td>0.39</td>
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<td></td>
<td>213.8</td>
<td>325.8</td>
<td>248.7</td>
<td>212.0</td>
<td>205.4</td>
<td>23.6</td>
<td>0.08</td>
<td>0.01</td>
<td>0.04</td>
<td>0.85</td>
</tr>
<tr>
<td>Ileum wk 8</td>
<td>262.2</td>
<td>265.9</td>
<td>395.1</td>
<td>362.5</td>
<td>268.3</td>
<td>45.5</td>
<td>0.31</td>
<td>0.09</td>
<td>0.83</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>281.5</td>
<td>279.3</td>
<td>276.6</td>
<td>279.8</td>
<td>286.1</td>
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<td>0.86</td>
<td>0.97</td>
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<td>0.64</td>
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<tr>
<td>Brush border membrane, milliunits/mg (wet tissue)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Duodenum wk 8</td>
<td>241.1</td>
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<td>231.6</td>
<td>8.6</td>
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<td>227.4</td>
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<td>0.04</td>
<td>0.49</td>
<td>0.02</td>
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</tr>
<tr>
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<td>253</td>
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<td>0.43</td>
<td>0.56</td>
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<td>42</td>
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<td>0.90</td>
<td>1.00</td>
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<tr>
<td>AA digestibility, %</td>
<td>Excreta wk 8</td>
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<td>76.4</td>
<td>76.7</td>
<td>75.5</td>
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<td>0.34</td>
<td>0.80</td>
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<tr>
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<td>Ileum wk 14</td>
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<td>89.1</td>
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<td>0.6</td>
<td>0.01</td>
<td>0.04</td>
<td>0.14</td>
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</table>

1Values are expressed as means (n = 5/treatment) of biweekly data over the whole period.

2DG = defatted green microalgal biomass (Desmodesmus spp.; Cellana, Kailua-Kona, HI); FD = full-fatted diatom biomass (Staurosira spp.; Cellana).

3Preplanned contrasts were I = control vs. DG and FD, II = DG vs. FD, III = DG vs. DG + protease, and IV = FD vs. FD + protease.


5Measured at wk 14 using Cr2O3 as an indigestible marker.

actually lower than those fed the control, indicating a potential of better status of bone health or phosphorus nutrition or both (Igarashi et al., 2002; Ekmay et al., 2012). Thus, both types of microalgal biomass supported hen performance and health when they were incorporated into the corn–soy–wheat basal diet at the designated rates on the isocaloric and isonitrogenous bases. The FD diet exerted different impacts on several egg quality traits and plasma TRAP activities than the DG diet. Adding protease to the DG and FD diets exerted variable effects on the weights of egg yolk or shell, plasma corticosterone concentrations, and plasma TRAP activities, but these effects were neither consistent nor strong.

In our earlier study (Leng et al., 2014), we fed Shaver laying hens with the defatted diatom microalgal biomass for 8 wk and observed decreased feed intake and egg production in hens fed 15% compared with those fed the control diet. Similarly, hens fed the 2 types of microalgal biomass had a lower overall feed intake. Their depressed feed intake or limitation on the dietary inclusion might be attributed to the high salt and ash or indigestible cell wall components (polysaccharides and algaenans) or both. However, the decreased feed intake did not cause any loss of egg production or BW, indicating that nutrients from the ingested DG or FD diets were more efficiently utilized by hens than those from the control diet.

Long-term studies such as those by Lipstein et al. (1980) and El-Deek and Al-Harthi (2009) successfully incorporated algae at levels above 10%, whereas short-term studies such as those by Ginzberg et al. (2000) and Lipstein and Hurwitz (1981) showed a depression in laying hen and broiler performance. Therefore, the extended feeding time in the present study allowed for a greater ability to demonstrate the potential for feeding the hens with these microalgal products. Moreover, this study extends our previous research on the defatted diatom microalgal biomass (Austic et al., 2013; Leng et al., 2014) to a new source of green microal-
gae. With 31% CP, the DG was incorporated at a much greater level (25%) than the diatom, with only moderately adverse effect on the feed intake or egg and egg albumen weights. Although the prevailing notion is that algae can only be included in the diet at up to 10% (Becker, 2004), our results indicate that greater levels of incorporation are possible given appropriate formulation of the diet.

In the present study, feeding DG or FD decreased concentrations of plasma 3-MH in the hens. As a biomarker of protein degradation (Young and Munro, 1978), the lower plasma 3-MH likely indicates less mobilization of muscle tissue to meet the protein requirements by the hens. Feeding DG or FD also consistently improved ileal total AA digestibility over the control. This result is contrary to the perception that algae can only be used in the diet up to 10% (Becker, 2004), but our results indicate that greater levels of incorporation are possible given appropriate formulation of the diet.

The lack of stronger or more consistent improvement of the total AA digestibility or intestinal protease activity by the supplemental protease remains unclear. There was no apparent association between intestinal protease activity and ileal AA digestibility. It has been shown that intestinal peptidase activity adapts to changes in dietary protein intake and quality (Corring, 1980). As all diets in the present study were formulated to be isonitrogenous, changes in intestinal brush border membrane proteolytic activity in the DG- or FD-fed hens over the controls might be attributable to differences in dietary protein quality (Gilbert et al., 2008, 2010), such as protein types and AA profile or balance (Smith and Young, 1955; Lewis and Gonzalves, 1960; Punnett and Derrenbacker, 1966; Boyd, 1973; Campanella et al., 2002). Meanwhile, the intestinal protease activities could be affected by dietary fiber. High dietary fiber upregulated small intestinal peptidase activity in weanling pigs (Hedemann et al., 2006). Farness and Schneeman (1982) reported an increased peptidase activity in the small intestine of rats fed high levels of pectin or cellulose. Aumaître and Lizardo (2001) found that the inclusion of non-starch polysaccharide from beet pulp in the diet of weanling pigs elevated peptidase activity in their intestines, with improved growth performance and car-

<table>
<thead>
<tr>
<th>Diet:</th>
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<td>-</td>
</tr>
<tr>
<td>Protein</td>
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<td>+</td>
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<th>FD</th>
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<td>1.00 ± 0.07</td>
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<td>0.88 ± 0.15</td>
<td>0.48 ± 0.07b</td>
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</thead>
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<td>1.09 ± 0.05</td>
<td>1.09 ± 0.05</td>
<td>1.07 ± 0.17</td>
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<th>PS6</th>
<th>Control</th>
<th>DG</th>
<th>FD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00 ± 0.06</td>
<td>5.84 ± 0.04b</td>
<td>7.79 ± 0.08</td>
<td>4.81 ± 0.05b</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PS6/S6</th>
<th>Control</th>
<th>DG</th>
<th>FD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00 ± 0.06</td>
<td>5.84 ± 0.04b</td>
<td>7.79 ± 0.08</td>
<td>4.81 ± 0.05b</td>
</tr>
</tbody>
</table>

Figure 1. Effects of dietary microalgal biomass and protease supplementations on hepatic protein levels of protein synthesis-related key regulators at wk 14. Values under bands of each group are expressed mean ± SE. *Different from the control, P < 0.10. †Different from the control, P < 0.05. ‡Different from the same treatment diet without protease, P < 0.10. §Different from the same treatment diet without protease, P < 0.05. DG = defatted green microalgal biomass (*Desmodesmus* spp.; Cellana, Kailua-Kona, HI); FD = full-fatted diatom biomass (*Staurosira* spp.; Cellana); eIF4E = eukaryotic initiation factor 4E; mTOR = mammalian target of rapamycin; P70 = P70 S6 kinase 1; S6 = S6 ribosomal protein; PS6 = phospho-S6 ribosomal protein. Protease was Ronozyme ProAct L (DSM Nutritional Products Inc., Parsippany, NJ).
Table 7. Effects of dietary microalgal biomass and protease supplantations on mRNA levels of AA and peptide transporters in the duodenum of hens at wk 14\(^1\)

<table>
<thead>
<tr>
<th>Gene(^2)</th>
<th>Control</th>
<th>DG</th>
<th>FD</th>
<th>Protease(^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Apn)</td>
<td>1.00</td>
<td>0.52</td>
<td>0.82(^b)</td>
<td>0.72</td>
</tr>
<tr>
<td>(Cat1)</td>
<td>1.00</td>
<td>0.76</td>
<td>1.43</td>
<td>1.57(^a)</td>
</tr>
<tr>
<td>(Lat1)</td>
<td>1.00</td>
<td>0.29(^a)</td>
<td>0.49(^b)</td>
<td>0.61</td>
</tr>
<tr>
<td>(PepT1)</td>
<td>1.00</td>
<td>1.43</td>
<td>1.92</td>
<td>0.12(^a)</td>
</tr>
</tbody>
</table>

\(\text{SEM}\)

\(^a\)Different from the control \((P < 0.05)\).

\(^b\)Different from the same treatment without protease \((P < 0.05)\).

\(^1\)Data are expressed as means of the relative changes compared to the control \((n = 3 \text{ to } 4)\).

\(^2\)\(Apn\) = aminopeptidase N; \(Cat1\) = cationic amino acid transporter-1; \(Lat1\) = L-type amino acid transporter-1; \(PepT1\) = peptide transporter-1.

\(^3\)DG = defatted green microalgal biomass \((Desmodesmus spp.; Cellana, Kailua-Kona, HI); FD = full-fatted diatom biomass \((S. dentiferum\text{ spp.}; Cellana).\)


cass composition. Therefore, the mixed responses of intestinal protease activities among the hens fed the DG, FD, or control diets might be confounded with their variable types or levels of fiber. Future research should explore if and how certain types of polysaccharides in the microalgal biomass, in particular, in the cell wall, affect intestinal proteolytic activity and ileal AA digestibility in the hens.

Both the inclusion of the 2 types of microalgal biomass and the inclusion of protease exerted mixed impacts on gene expressions of the duodenal AA and peptide transporters and \(Apn\) at wk 14. Although Gilbert et al. (2007) found increased expression of \(PepT1\) because of higher protein quality, we saw a downregulation of the gene by feeding FD and a restoration by adding protease to the FD diet. Likewise, the mRNA level of \(Lat1\) was decreased by feeding DG, and partially restored by adding protease into the diet. In contrast, the mRNA level of \(Cat1\) was elevated by feeding FD and decreased by adding protease in the diet. Expression of \(Cat1\) in the jejunum was decreased in growing pigs fed a wheat-based diet supplemented with Lys, Thr, and Met (Garcia-Villalobos et al., 2012). However, Gilbert et al. (2010) did not show any changes in these 2 genes because of protein composition. Although lower expression of \(Apn\) indicates a greater efficiency of dietary protein (Speier et al., 2012), adding protease to the DG diet actually elevated its mRNA in the present study.

Notably, feeding DG and FD upregulated S6 phosphorylation and the ratio of PS6:S6 in the liver of hens, compared with the control diet. Because elevated phosphorylation of S6 promotes mRNA translation (Everaert et al., 2010), our results demonstrated a novel, unique potential for both types of microalgal biomass in stimulating liver protein synthesis. That feature was consistent with their ability to improve plasma 3-MH concentrations and ileal AA digestibility. However, the upstream regulators of S6 phosphorylation (P70 and mTOR) showed little or negative responses to the microalgal biomass and protease feeding. This may imply alternative mechanisms for stimulating S6 phosphorylation by DG and FD. Indeed, Duchêne et al. (2008) reported that S6 phosphorylation did not occur through the S6K1 cascade in the liver of chickens but most likely occurred through an alternative pathway. In addition, neither the microbial biomass nor protease exerted major effects on hepatic eIF4E. Apparently, caution should be given to interpret our molecular data on gene expression and signal proteins because of the lack of globally consistent responses to either microalgal biomass and protease or direct correlation with the growth performance and biochemical status. Overall, our results show that feeding the layer hens with the corn–soy–wheat diets containing 25% DG or 11.7% FD for an extended period of 14 wk did not produce adverse effects on egg production or body health. In fact, inclusions of the 2 types of microalgal biomass exerted a number of positive effects on dietary AA digestibility and body protein metabolism. However, the biochemical and molecular mechanism for their positive effects and their interactions with exogenous proteases requires more systematic research.

**LITERATURE CITED**


